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## **In-Vitro Demonstration of Cell-Mediated Immunity to Vaccinia Virus in Man**

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### **Abstract**

Cell mediated immunity to vaccinia virus in man was studied by lymphocyte transformation. Vaccinia antigen, propagated on BHK-21 and Vero cells, could be used successfully for in-vitro testing after partial purification as well as crude infectious homogenates. Vaccinia antigen preparations were effective both in the infective and the inactivated state. Inactivation was usually accompanied with a certain loss of stimulating activity. Development of cell mediated immune response in-vitro after first vaccination was investigated in 17 adults. Vaccinia virus specific lymphocyte transformation was seen in the second week after vaccination in all cases. Following revaccination no increase of lymphocyte transformation ratio could be observed in 11 persons studied. At the same time the titers of humoral antibodies were elevated.

### **Introduction**

Host response to viral infections include humoral and cellular immune mechanisms. Recovery from viral infections may be dependent on antibodies; antibody formation is of undoubted value in preventing disease (3). However, the development of delayed hypersensitivity to viral antigens is well recognized and at least in some viral diseases recovery from the primary infection may be largely because of cellular immune mechanisms (2). In vaccinia infection it was supposed from early studies of v. PIRQUET (17) that cellular immunity might be of essential importance. In experimental vaccinia infection, impairment of host cell-mediated responsiveness by use of immunosuppressive agents increased morbidity and mortality (11). In humans progressive vaccinia occurred in subjects with defects of cell-mediated immunity (8). Thus measurement of antibodies seems not to be sufficient enough for the evaluation of the immune status of a vaccinated person. Cell mediated immunity (CMI) to vaccinia virus has been successfully demonstrated in animals (7, 9, 15, 16, 19). In humans CMI to vaccinia has been tested by intracutaneous application of antigen (14) and also in-vitro by using different assays (5, 10, 12, 20, 21, 24). In comparing the results difficulties may arise because the immune response of a vacci-

nated individual to vaccinia antigen in-vitro may to some extent depend on the host cell used for preparation of the in-vitro test antigen (1). We therefore investigated the immune response of vaccinated persons to infectious and inactivated vaccinia virus antigen by using the lymphocyte-transformation test. Secondly we investigated the onset of cell-mediated reactions in-vitro following primary vaccination and the possible booster reaction of cellular immunity after revaccination.

## Material and Methods

### Subjects

17 individuals, ranging in age from 17–28 years were studied for immune reactions after a primary vaccination. These subjects were volunteers, who required vaccination in order to obtain an international travel certificate. These persons had not received vaccination in childhood because of cutaneous or other illnesses undergone by themselves or other members of their families. Eleven individuals of the institute staff were checked for cellular and serological immune reactions after revaccination.

### Viruses and cell cultures

The following strains of viruses were used for preparation of in-vitro antigens: Vacciniavirus strain WR with titers of  $10^{6.5}$  TCID<sub>50</sub>/ml, Vesicular stomatitis virus (VSV) strain Indiana ( $10^6$  TCID<sub>50</sub>/ml). Titration of vaccinia virus and VSV was performed on Vero cells.

Cell cultures were grown and maintained in Eagle's minimum essential medium supplemented with 15% calf serum, 100 units of penicillin/ml and 100 µg/ml streptomycin. Viruses were propagated on permanent monkey kidney-cell cultures (Vero) or on permanent baby hamster kidney-cell cultures (BHK-21). – For vaccination vaccinia virus strain Elstree pfu  $1 \times 10^8$ /ml (Landesimpfamt Düsseldorf) was used. In the case of primary vaccination 1000 IU anti-vaccinia-globulin (Vacciniabulin®/Immuno) was applied.

### Preparation of antigens

The infected cell cultures were disrupted by freezing and thawing 3 times after titration, the cell homogenate was sonicated  $3 \times 10$  sec with a Branson Sonifier at stage 4. The material was centrifuged for 20 minutes at 2500 rpm/4°C to remove rough cell fragments. The supernatant was subjected to a pellet centrifugation in a Beckmann centrifuge L2 at 4°C, 90 minutes 17000 rpm in the rotor SW 27 or type 19 120 minutes, 18000 rpm. In these steps the volume was reduced 100 : 1. The pellet was suspended in phosphate buffered saline pH 7.2 sonicated again for 10 seconds and subsequently purified from cell fragments by 5 minutes 5000 rpm 4°C centrifugation in an IEC-centrifuge. The TCID<sub>50</sub>/ml of this preparation reached usually  $1 \times 10^8$ – $1 \times 10^9$ .

### Virus inactivation

The infected cell homogenate or partly purified material was inactivated with formalin 1 : 4000 over 24 hours at 37°C. Abundant formalin was bound with sodium pyrosulfite in equimolar concentration. UV-inactivation was performed by irradiation of minute volumes in a thin layer in petri dishes for 5 minutes at a distance of 10 cm from the UV-source (output 10 Watt at 254 nm).

### Lymphocyte transformation-test

Blood was aseptically removed and mixed with heparin (25 U/ml). Sedimentation of erythrocytes was performed after addition of 1 ml 5% Dextran (MG 250,000, Pharmacia, Uppsala) to 9 ml blood and incubation in 45° inclined tubes for 45 minutes at 37°C. The upper phase, rich in lymphocytes was washed 2 times in Hanks balanced salt solution, containing 2% inactivated fetal calf serum and 5 U heparin/ml and then suspended in culture medium (TCM 199 with 15% inactivated fetal calf serum, 100 U penicillin and 100 µg streptomycin) to give a final concentration of  $2 \times 10^6$  cells/ml. Cultures were set up in plastic tubes (Greiner, Nürtingen, No. 1608) containing 1 ml lymphocyte suspension and 0.2 ml of antigen: viral antigen, noninfective tissue cell homogenate or control antigen of VSV to which the patient was not sensitized. The ability for unspecific transformation was tested by addition of Phytohemagglutinin (Wellcome) 12.5 µg/ml. Each test was run at least 3 times. The tubes were incubated at 37°C for 5 days in a humidified 5% CO<sub>2</sub> atmosphere. Twelve hours before the termination of cultures 1.5 µCi <sup>3</sup>H-Thymidine (spec. activity 23 mCi/mmol, Radiochemicals Amersham, Buchler, Braunschweig) were added. To terminate the cultures the tubes were cooled to 4°C, centrifuged at 1000 rpm for 10 minutes, and the supernatants discarded. The sediments were suspended in 10 ml physiological NaCl, filtered through membrane filters, pore diameter 0.2 µ (SARTORIUS, Göttingen; Cat. No 11307), and washed with 10 ml ice cold 5% trichloroacetic acid (TCA). The dried filters were put into vials with scintillation fluid and counted in a Liquid scintillation spectrometer (Packard Model 3380). Transformation ratio was calculated as the relationship of incorporation of radioactivity in acid insoluble DNA in antigen transformed cultures to control cultures.

### Neutralization test

Sera assayed for vaccinia antibody were heated at 56°C for 30 minutes before assay and serially diluted in fourfold steps in Eagle's MEM. Mixtures, containing 2 parts of diluted sera, 1 part of guinea pig complement (90 hemolytic U/ml, 1:15 diluted prior to use) and 1 part of virus suspension (giving a final virus concentration of 100 TCID<sub>50</sub>/0.2 ml of mixture) were incubated overnight at 37°C. Aliquots of 0.2 ml/well were then inoculated on monolayers of Vero cells, prepared in microtiter plates (Greiner, Nürtingen; No. M220-29 ART). Each serum dilution was tested fourfold. Controls were run for the calculation of the TCID<sub>50</sub>/ml without additions, other controls contained guinea pig complement and virus without antibodies. Human antivaccinia immune serum (Vaccinia-bulin®/Immuno) served as standard in each test. Neutralization units NU 50 were counted according to the method of REED and MUENCH (18) after 48 hours.

## Results

### Optimal incubation time and dosis – optimum of antigen

The incubation time of culture required to give an optimal transformation-ratio at a given time (day 20–25) after vaccination was assayed in primary vaccinated volunteers (N = 5). Augmented <sup>3</sup>H-Thymidin incorporation could already be observed after 72 hours and reached a maximum after 120 hours of incubation, later on the incorporation ratio declined rapidly. Infectious partially purified vaccinia virus was used as test antigen (Fig. 1). For every preparation of viral

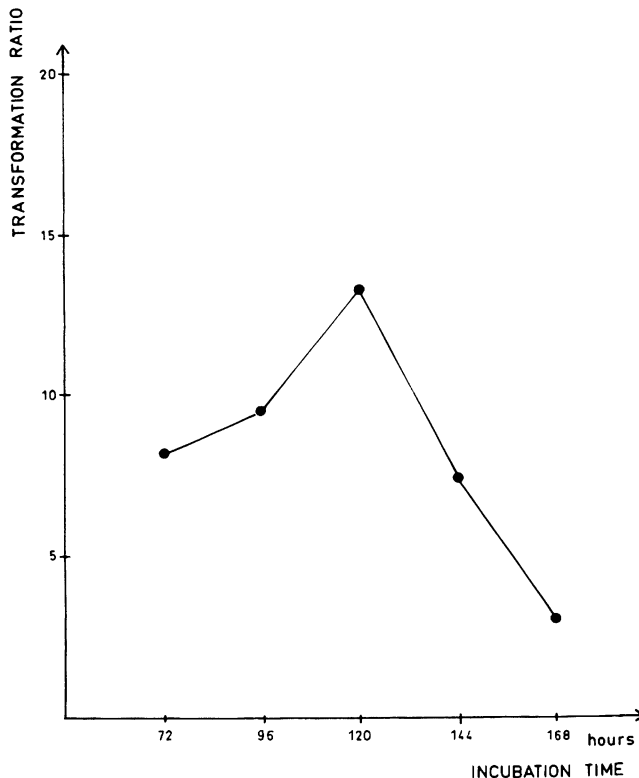


Fig. 1. Transformation ratios at different times after adding 0.2 ml partially purified infectious vaccinia virus antigen. Mean values of ( $N = 5$ ) lymphocyte donors 20–25 days after primary vaccination.

antigen the dosis-optimum had to be determined. Figure 2 shows the dependence of transformation-ratio on antigen-dilution using a non-purified infectious cell homogenate (VZ9). Whilst concentrated material (protein content 1.5 mg/ml) showed no activity, transformation-ratios increased with dilution of the antigen up to an optimum at a dilution of 1 : 10. Partially purified vaccinia antigen on the other hand induced specific transformations in the undiluted state.

#### Specificity of virus induced lymphocyte transformation

To confirm the virus-specificity of the transformation, transformation-ratios induced by vaccinia antigen (both infectious and UV-inactivated) and 2 control antigens, vesicular stomatitis virus (VSV) and a Vero-cell homogenate were compared. VSV-antigen was used in a concentration that was effective in inducing transformation of lym-

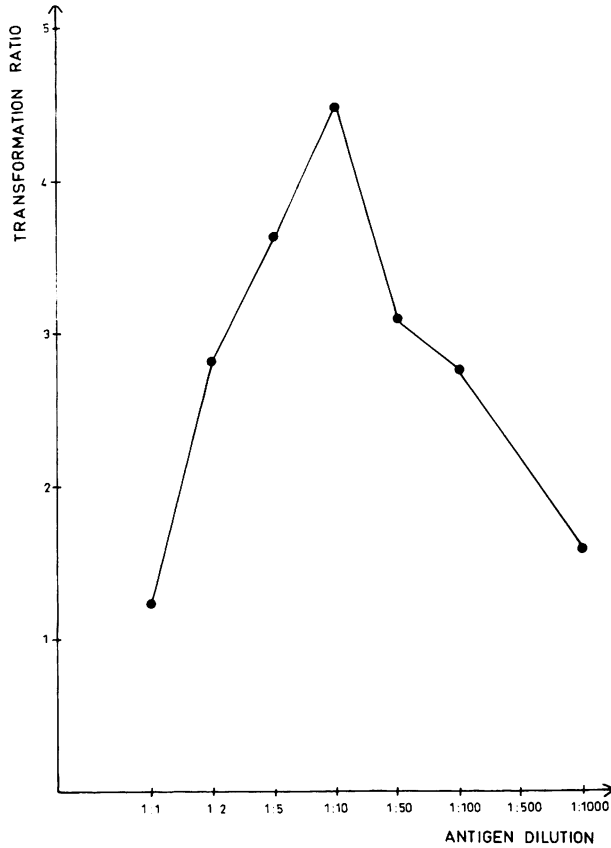


Fig. 2. Transformation ratios of crude vaccinia virus infected tissue culture material (Vero-cells, preparation VZ9). Toxic effects of concentrated material can be removed by dilution in tissue culture medium. Lymphocyte donors ( $N = 5$ ), 20–25 days after primary vaccination.

Tab. 1. Specificity of vaccinia virus induced lymphocyte transformation

Test No.	control without antigen	Vaccinia (infectious)	Vaccinia (UV inactivated)	Vesicular Stomatitis Virus (infectious)	Vero-cell antigen control
25	171	1201*	n.t.	35	168
27	151	778**	n.t.	192	85
40	188	1470*	474**	191	n.t.
41	150	1364*	795*	164	145

mean of 5 values, d.p.m.

\* < P 0.01 compared with antigen free control and Vero-cell antigen control

\*\* < P 0.05 compared with antigen free control and Vero-cell antigen control

n.t.: not tested

phocytes from VSV-sensitized guinea pigs in-vitro as shown by earlier experiments (13). The Vero-cell control, equal in protein content to the vaccinia-virus preparation, was run, because the test antigens had been propagated on Vero-cells. As shown in Table 1, only vaccinia antigens were able to transform lymphocytes of vaccinia-sensitized persons.

### Transformation induced by different vaccinia antigen preparations

Different vaccinia virus preparations harvested from BHK-21 and Vero-cells were tested as in-vitro antigens. Unrelated to protein content or TCID<sub>50</sub>/ml the harvests differed widely in their transforming activity, whereas inactivation was always accompanied with a certain loss of in-vitro transforming activity (Fig. 3).

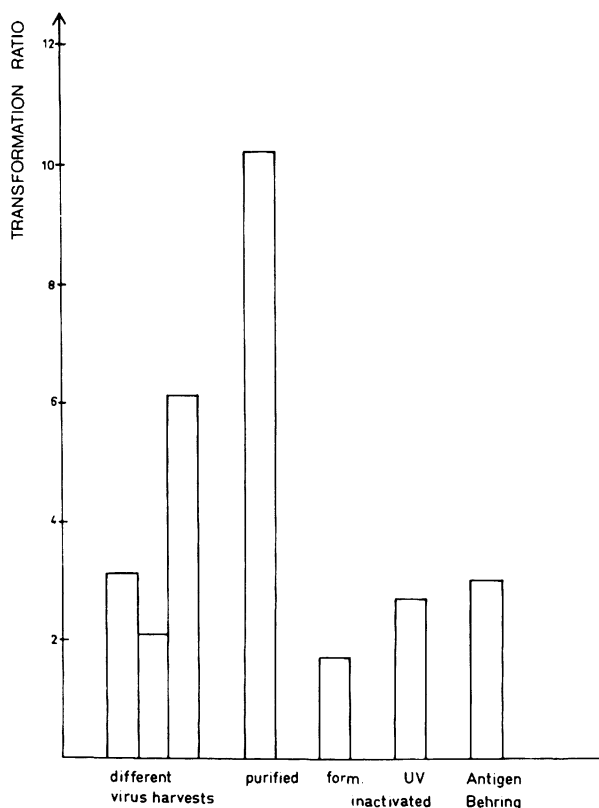


Fig. 3. Transformation activities of different vaccinia virus antigen preparations, propagated on BHK-cells. Lymphocyte donors ( $N = 8$ ). 20–25 days after primary vaccination. Antigen 0.2 ml, 1 : 10 diluted in medium, incubation time 120 hours.

### Virus specific in-vitro transformation after primary vaccination

17 healthy volunteers were vaccinated with vaccinia virus strain Elstree with simultaneous injection of 1000 IU anti-vaccinia globulin. On the day of vaccination and 4 times later at intervals of 4–5 days blood was taken for assay of lymphocyte transformation with vaccinia antigen (antigen VZ9 propagated on Vero-cells was used throughout this study). The formation of antibodies was tested in parallel. Each test person developed significant vaccinia virus specific transformation. The first specific transformations were seen in the second week after sensitization (Fig. 4). The onset of in-vitro transformation and specific cutaneous reactions did not correlate, the latter were always 3–5 days positive before the beginning of specific lymphocyte transformation. At the time of maximal transformation of lymphocytes no antibodies to vaccinia could be detected by neutralization test.

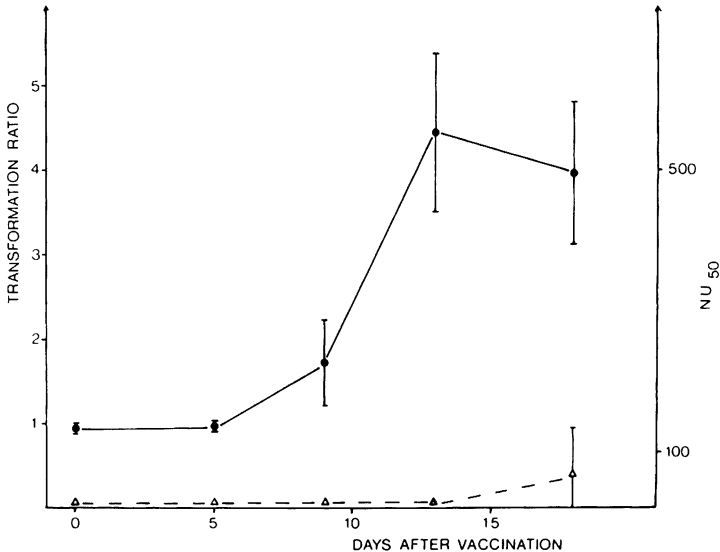


Fig. 4. Development of cell mediated immune response (straight line) and of neutralizing antibody formation (dashed line) after primary vaccination ( $N = 17$ ). Antigen VZ9, incubation time 120 hours.

### Immune reactions after revaccination

11 healthy volunteers who had already been vaccinated within the last 1–7 years were revaccinated. All persons had established cell-mediated immunity to vaccinia virus and no booster effect on in-vitro reactivity after revaccination was observed (Fig. 5). Simultaneously



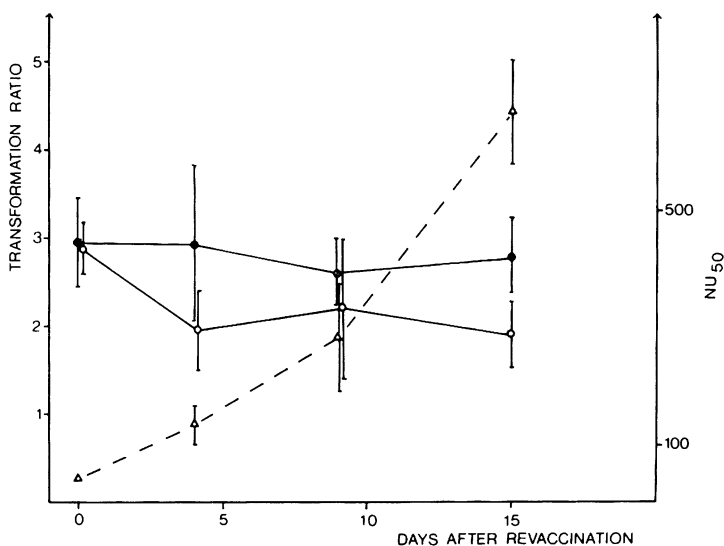


Fig. 5. Transformation ratios in lymphocyte cultures after revaccination.

a) ● Lymphocyte donors with specific cutaneous reactions ( $N = 11$ ).

b) ○ Lymphocyte donors without specific cutaneous reactions ( $N = 5$ ).

Line with dashes: increase of neutralizing antibodies persons successfully re-vaccinated (a).

the sera of those persons who had shown positive cutaneous reactions were tested for neutralizing antibodies. Although there was no augmentation of CMI in-vitro, an increase of the antibody level to vaccinia could be observed.

## Discussion

In persons sensitized to vaccinia virus, cell mediated immunity can be demonstrated by lymphocyte transformation test with a good reproducibility. The results are virusspecific and unspecific cross reactions with an unrelated virus were not seen. Lymphocytes from persons without prior contact to vaccinia virus in no case developed positive in-vitro reactions. The incubation time to obtain optimal transformation with vaccinia antigen corresponds well with the incubation time using other bacterial, cellular and viral antigens for demonstration of CMI in-vitro. Difficulties may appear in the choice of antigen for lymphocyte transformation. In our system commercial vaccines did not work well. Moreover, by using the same vaccine as antigen for in-vitro testing, as used for vaccination, there may be some interference of virus specific immune reactions with cell mediated immune response directed against antigens on the host cell used for virus

propagation. In experiments with laboratory animals, vaccinia virus worked as an immunological adjuvant to host cell transplantation antigens (1). Therefore, as commercial vaccines were propagated in calves the test antigens were prepared from virus grown in tissue culture of different origin. The harvests differed significantly in their transforming activity not dependent on virus and protein content. Purification of virus particles did not always lead to higher blast formation. Not only viral particles alone, but virus induced cell surface antigens may also be capable of inducing cell mediated immune reactions in-vitro (22).

Since some viruses can transform and replicate in lymphoid cells or depress lymphoid immune reactions (4) the use of inactivated vaccines for in-vitro testing might be advantageous. In our experiments, working with optimal antigen concentrations, we never saw an unspecific inhibitory effect of infective vaccinia virus. There is little known about the onset of delayed type immune reactions after primary vaccination. While several authors could show lymphocyte transformation to vaccinia in immune human subjects (6, 10, 12, 24) there exists only one communication concerning the time course of development. By microscopic counting of blast cells augmented lymphocyte transformation after antigenic challenge in man was observed 20 days after vaccination (20). In vaccinia injected animals enhanced transformation reactions to vaccinia were already seen after 5 days, reaching a maximum in the second week after sensitization (19). In experiments with guinea pigs (13) and rabbits (unpublished data) we also found increased transformation ratios at earlier stages. Delayed type vaccinia-specific cutaneous reactions always preceded the onset of positive in-vitro transformation reactions. This difference may represent the time needed for sensitized cells to reach the circulation: Depending on dose and way of antigen application active lymphocytes can be found first in regional lymphnodes, then in the spleen and several days later in the peripheral blood (23). In experiments with rabbits (19) optimal transformation reactions could be demonstrated only within a relatively short time range after immunization. The time of harvest of lymphocytes for in-vitro investigations may therefore be very important. The early peak of lymphocyte response can also be observed in their effector functions, by testing cytotoxic activities on virus infected target cells (9). It remains to be determined, what kind of cells are responsible for the immune response later after infection.

In contrast to the well known second set reaction in transplantation experiments we found, apart from an increase of neutralizing antibodies, no significant differences in the proliferation of lymphoid cells of vaccinia immune donors after revaccination. We suggest, that in immune persons, who show specific blast transformation, the effects of revaccination on delayed hypersensitivity may be restricted to the

draining lymph nodes and that the generalized effects are small and, unlike the first vaccination, escape the lymphocyte transformation test. Similar results have been obtained by other authors looking for interferon production after revaccination (6). While there was an increase of interferon production in-vitro following addition of vaccinia antigen, no significant alteration of lymphocyte transformation after revaccination could be observed.

Hence it may be of doubtful value to use the lymphocyte transformation test to quantify the vaccinia virus immune status for clinical purposes. However, it may be a useful test in looking for CMI in diseases with impaired delayed hypersensitivity or in cases of uncertain cutaneous and serological reactions after primary vaccination.

### Acknowledgement

We thank Mrs. G. KÖHLER for skillful technical help.

### Zusammenfassung

In-vitro-Nachweis zellulärer Immunität gegen Vacciniavirus beim Menschen

Zellvermittelte Immunität gegen Vacciniavirus wurde in vitro durch die Lymphozytentransformation menschlicher Lymphozyten untersucht. Vaccinia-Antigen, vermehrt auf BHK-21 und Vero-Zellen, konnte mit Erfolg im teilweise gereinigten wie auch im ungereinigten Zustand für die In-vitro-Testung verwendet werden. Vaccinia-Antigen-Präparationen waren sowohl im infektiösen als auch im inaktivierten Zustand wirksam, die Inaktivierung war gewöhnlich von einer gewissen Einbuße an stimulierender Aktivität begleitet. Die Entwicklung der zellvermittelten Immunantwort nach Erstimpfung wurde bei 17 Erwachsenen untersucht. Vacciniavirus-spezifische Lymphozytentransformationen konnten in der zweiten Woche nach der Impfung in allen Fällen beobachtet werden. Elf geimpfte Personen wurden hinsichtlich ihrer Immunantwort nach Wiederimpfung untersucht. Während die neutralisierenden Antikörper anstiegen, war eine Zunahme der Lymphozytentransformationsrate nicht nachweisbar.

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